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ORIGINAL PAPER

Antibody responses in human cystic hydatid disease to the laminated layer of *Echinococcus granulosus*

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Abstract The laminated layer of hydatid cysts of *Echinococcus granulosus* represents a considerable amount of parasite material. Its antigenic role, however, is unclear. Extracts of laminated layer taken from sheep cysts were analysed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and were found to contain bands at 55 and 25–29 kDa, which reacted with an anti-sheep IgG antibody probe, indicating that these were likely to be host-contaminating components within the layer. However, the same bands were also recognised by a significant proportion of human hydatid patients, particularly by IgG4 antibodies, and not by negative control individuals. These individuals did not recognise immunoglobulin heavy and light chains in a sheep serum extract in the same manner. It seems likely that there are either host or parasite antigenic components at similar molecular weights or that certain parasite antigens may share epitopes with sheep immunoglobulins. The antigens at 25–29 kDa were found to be glycoproteins by lectin blot analysis and may be important markers of disease status.

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Introduction

Cystic hydatid disease, caused by the cestode *Echinococcus granulosus*, is characterised by the presence of slow-growing, unilocular cysts in various organs of the intermediate host. The disease is frequently accompanied by a Th2 type immune response, which is permissive of parasite survival (Ortona et al. 2003). The cysts are fluid-filled and have a living germinal layer, which produces protoscoleces, and an extensive, non-living laminated layer, which is largely a protein/carbohydrate matrix. The laminated layer is the outermost region of the cyst and provides a degree of both mechanical and immunological protection for the germinal layer, in that it may form a barrier against cellular attack (Rickard and Williams 1982; Dixon and Jenkins 1995a,b; Rogan and Craig 1997; Steers et al. 2001; Gottstein et al. 2002). This layer is not, however, a barrier to antibody as immunoglobulins have been detected within the cyst fluid in some host species (Coltorti and Varela-Diaz 1972; Edwards 1982).

In large cysts in humans, the laminated layer may be several millimetres thick and, therefore, represents a considerable mass of parasite tissue. Its antigenicity, however, has not been fully elucidated. However, there has been considerable recent interest in the role of parasite carbohydrates and glycoproteins in modulating the immune response. In *Echinococcus multilocularis* where the laminated layer is less extensive, significant antigenicity has been detected. The antigen Em2 has been particularly identified as having diagnostic importance for human alveolar hydatid disease (Gottstein et al. 1983; Gottstein 1985; Hulsmeier et al. 2002). This is a 54 kDa mucin-type, glycoprotein antigen, which shows no cross-reaction with sera from cystic hydatid patients. The laminated layer of *E. granulosus* has received less attention because the major diagnostic anti-

gens for this species are found in the hydatid cyst fluid. However, Bigute et al. (1962) and Varela-Diaz and Torres (1977), using precipitation techniques, have reported common parasite antigens between whole cyst membranes (laminated layer and germinal layer), cyst fluid and protoscoleces of *E. granulosus*. Russi et al. (1974) isolated a glycoprotein antigen from intact membranes (laminated layer and germinal layer), which reacted with 11 out of 21 sera from human hydatid patients. In addition, Taherkhani (1998) found that 26% of hydatid patients from Turkana, Kenya recognised a laminated layer extract in the enzyme-linked immunosorbent assay (ELISA). It is not clear in any of these studies whether potential antigens are derived from the laminated layer or germinal layer because these are difficult to separate. Because this layer is the outermost part of the hydatid cyst, it is also in contact with host molecules and it is not clear to what extent isolated fractions of laminated layer contain host components.

In *E. multilocularis*, Em2 acts as a T cell independent antigen, which is important in parasite survival by modulating the host immune response (Dai et al. 2001). Because the quantity and carbohydrate nature of this layer in *E. granulosus* is more extensive, it could also be important in influencing the immune response in cystic echinococcosis. Recent studies have indicated that glycoproteins associated with parasite antigens are important in inducing a Th2 type immune response in chronic infections. Carbohydrate components have also been implicated in bringing about immunosupression via macrophages and other accessory cells. The significance of the carbohydrate-rich laminated layer in the immune response to *E. granulous* is therefore important. The aim of the current study was to identify which components of the laminated layer are of parasite origin and are recognised by antibody responses in natural human infections.

Materials and methods

Parasite material

Viable hydatid cysts were obtained from the livers and lungs of sheep slaughtered at a local abattoir. The hydatid fluid was aspirated from the cysts and the cyst wall, containing the laminated and germinal layers, was aseptically removed from the surrounding host capsule using forceps. The wall was cut into 1 cm strips, washed in sterile phosphate buffered saline (PBS) and frozen at −20°C overnight. The following day, this tissue was thawed and the germinal layer removed by peeling with forceps and scraping with a scalpel under a dissection microscope. The freeze/thaw process loosens the adhesion of the germinal layer to the laminated layer. Two ml packed volume of laminated layer was then added to an equal volume of PBS in a glass homogeniser and homogenised until only small fragments of laminated layer were visible. The preparation was then transferred to a 150 W sonicator and further disrupted for 15 min on ice using 10 s on and 10 s off cycles. The final preparation, which resembled a milky suspension, was stored at −20°C until required.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Parasite extracts and sheep serum (1/200 dilution) were run on 15% polyacrylamide mini-gels under reducing conditions. Briefly, 50 μl of laminated layer suspension or diluted sheep serum was added to 150 μl of sample cocktail containing 1.5 M dithiothreitol and boiled for 3 min. The preparation was then centrifuged in a microfuge at 5,000 rpm for 5 min and 20 μl added per lane of the gel. Gels were run at 25 mA/gel and either stained with Coomassie Blue or blotted on to nitrocellulose paper.

Immunoblotting

Proteins were transferred from gels to nitrocellulose paper (Sartorius 0.45 μm) by electrophoretic transfer in a Hoeffer Miniblotter at 100 mA/gel for 2 h. The nitrocellulose paper was blocked using 5% skimmed milk in 0.3% Tween 20 for 1 h, washed in PBS/0.1% Tween and probed with various sera either in trays or in a multi-track incubating chamber (Biometra). Human sera from individuals with confirmed hydatid disease from Turkana, Kenya and from those free of infection were added at 1/100 dilution in PBS/0.1% Tween. Blots were either probed with alkaline phosphatase-labelled anti-human IgG (Sigma) at 1/6,000 dilution or mouse anti-human IgG4 (Oxoid-Unipath) at 1/2,500 dilution, followed by alkaline phospahatase-labelled anti-mouse IgG (Sigma) at 1/20,000 dilution. Some fractions of laminated layer and sheep serum were also directly probed with alkaline phospahatase-labelled anti-sheep IgG (whole molecule) at 1/30,000 dilution. Blots were developed with bromochloroindoyl phosphate/nitro-blue tetrazolium (Sigma).

Lectin blotting

SDS/PAGE gels were blotted as above and blocked in Tris buffered saline (pH 7.4) containing 1% bovine serum albumen (BSA) for 1 h at room temperature. The nitrocellulose strips were then washed 3×10 min in TBS and incubated in lectin–peroxidase conjugates (Sigma) at 50 μg/ml in TBS for 1 h on a rocking platform. The nitrocellulose was then washed 3×10 min in TBS containing 0.1% BSA and once in TBS without BSA. Lectin conjugates were visualised by incubation in 0.05% diaminobenzidine (DAB)

containing 0.01% H_2O_2 in TBS for 5 min. The lectins used were concanavalin A (ConA), wheat germ agglutinin (WGA), soybean agglutinin (SBA) and horse gram agglutinin (DBA).

Results

The crude extract of sheep laminated layer in SDS/PAGE was shown to contain major bands at 55, 29, 27 and 25 kDa and minor bands at 200, 180, 97, 66, 42, 38, 36, 34, 17 and 15 kDa (Fig. 1). Samples of sheep hydatid cyst fluid had major bands at 66, 55, 29–27 kDa and minor bands at 38, 23, 17 and 12 kDa (Fig. 2). To try and identify potential host components in the parasite extracts, blots were probed with anti-sheep IgG whole molecule. An intense reaction was seen at 55 and 29 kDa in the laminated layer and sheep serum, but only at 55 kDa for the hydatid fluid (Fig. 3) presumably representing the presence of heavy and light immunoglobulin chains in these extracts. The 25 kDa band of the laminated layer was also labelled by this antibody but a similar band was not seen in the sheep serum sample. These results indicate that sheep laminated layer contains sheep IgG heavy and light chains at 55 and 29 kDa, respectively.

When blots of laminated layer were probed with a pool of human sera positive for hydatid disease, total IgG responses showed considerable binding in the 66–55 and

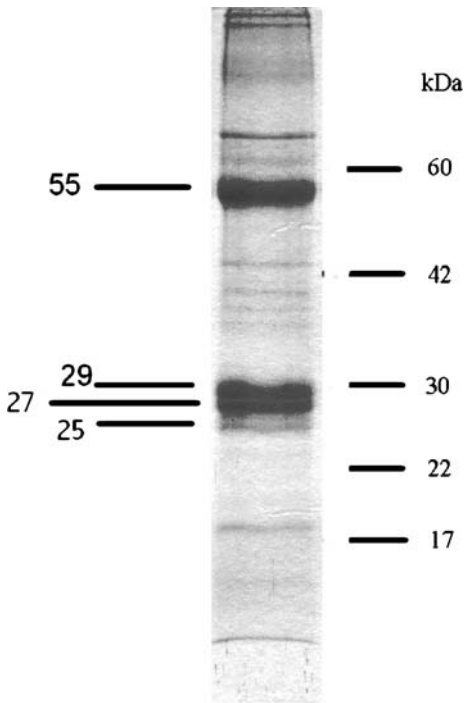

Fig. 1 SDS/PAGE profile of a sheep laminated layer extract stained with Coomassie Blue under reducing conditions. Significant bands are indicated

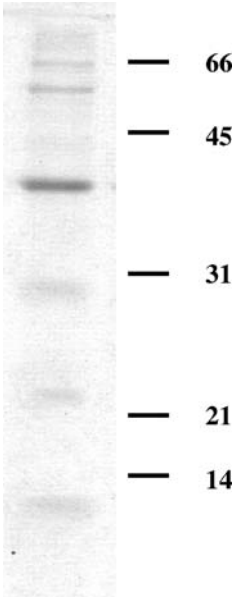

Fig. 2 SDS/PAGE profile of sheep hydatid fluid stained with Coomassie Blue under reducing conditions

29–25 kDa regions. The reactivity in this latter region was more intense than that obtained with a pool of control, uninfected human sera, particularly at 29 and 25 kDa although there was some background staining with the control negative serum. A sample of sheep serum was also run and probed with the same sera. In this case, the binding in the 66–55 kDa region was similar to that of the laminated layer, but the binding at 29–25 kDa was less intense than the laminated layer (Fig. 4). These results suggest that positive human sera recognises components at 29–25 kDa in the laminated layer in a different manner to potentially contaminating sheep serum molecules within the extract.

When individual human sera, rather than a pool, were tested against the laminated layer, total IgG responses showed moderate staining for both negative and positive individuals in the 66–50 kDa region, the 29–30 kDa region

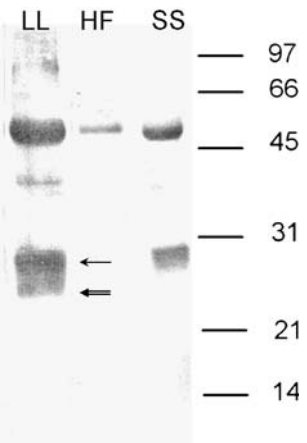

Fig. 3 Immunoblot of sheep laminated layer (*LL*), sheep hydatid fluid (*HF*) and sheep serum (*SS*) probed with anti-sheep IgG (whole molecule). Note that there are bands at approximately 40 and 25 kDa, which are recognised in the laminated layer but not in the sheep serum

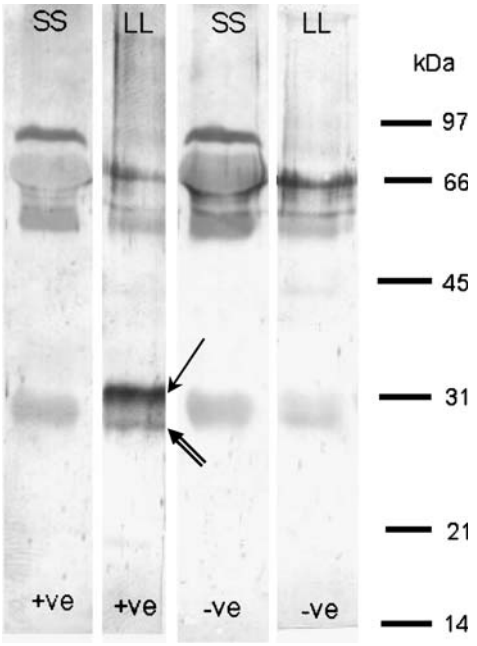

Fig. 4 Immunoblot of sheep laminated layer (*LL*) and sheep serum (*SS*) incubated with a pool of hydatid positive (*+ve*) and a pool of hydatid negative (*–ve*) human sera and then probed with anti-human IgG. Note that there is little difference between the positive and negative sera in recognising components in sheep serum. But that the positive serum recognises the 29 (*leftwards arrow*)–25 (*leftwards black arrow*) kDa region of the laminated layer to a greater degree than the negative serum

and the 17 kDa band, indicating general background activity in these regions (Fig. 5) (note that the anti-human IgG antibody conjugate control showed light–moderate staining in the 55 and 29–25 kDa regions, indicating probable cross-reactivity with sheep IgG). In 37 out of 55 positive cases (67%) however, at least 1 of the 3 bands in the 29–25 kDa region showed intense activity (Fig. 5). None of the 22 negative sera showed strong recognition of this region.

To eliminate background activity with human IgG probes, a monoclonal antibody to human IgG4 was used. When blots were probed for IgG4 activity, the background levels of staining were much reduced and negative sera showed very light staining in the 55 and 29–25 kDa regions (Fig. 6.). The positive sera, however, often showed intense staining with 42% strongly recognising the 55 kDa region and 77% strongly recognising 1 of the 3 bands in the 29–25 kDa region (Fig. 6). A pool of positive sera probed for IgG4 activity against the laminated layer and a sheep serum sample showed only light background at 66, 55 and 29–25 kDa in the sheep serum. But intense activity at the 29–25 kDa region for the laminated layer (Fig. 7), indicating that it was not sheep Ig light chain, which was being recognised in the laminated layer. Similar pools of sera were also used to probe a hydatid cyst fluid sample (Fig. 7). In this sample, the diagnostic bands of antigen 5 (38 kDa) and antigen B (10–24 kDa) were recognised by the positive serum and also the band at 29 kDa was weakly recognised in hydatid fluid antigen. However, a band at 25 kDa, similar to that of the laminated layer, was not recognised. Lectin blotting (Fig. 8) indicated that the bands at 55 and 29–25 kDa were glycoproteins containing mannoside (55, 29 and 27 kDa), glucosamine (29 and 27 kDa) and galactosamine (55, 29, 27 and 25 kDa) residues.

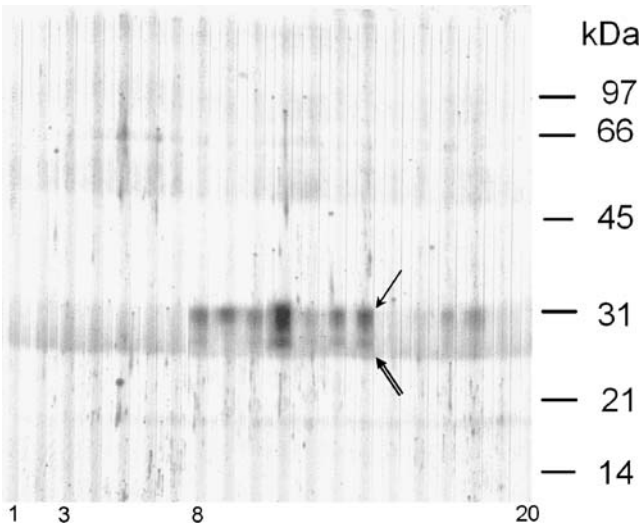

Fig. 5 Immunoblot of individual human sera against laminated layer extract, probed for total IgG activity. *Lanes 1–2*, anti-human IgG conjugate only; *lanes 3–7*, individual negative human sera; *lanes 8–20*, individual positive sera. Note that there is general background staining for all samples in the 66–50 and 29–25 kDa regions but that a number of positive samples have strong recognition in the 29–25 kDa region

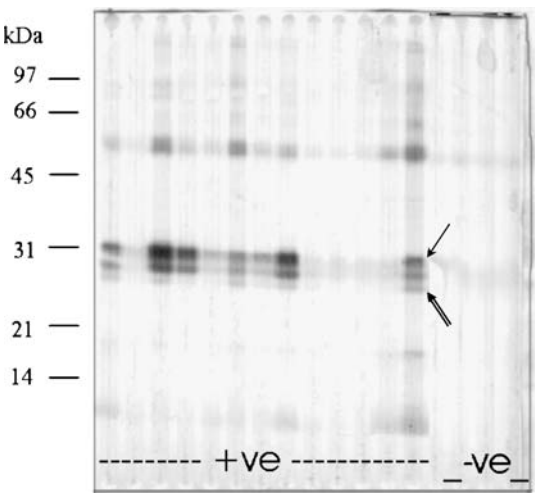

Fig. 6 Immunoblot of individual human sera against laminated layer extract, probed for IgG_4 activity. The first 13 sera are from hydatid patients (not the same sera as in Fig. 5) and the last 3 sera are from control negative individuals. Note that the staining for the negative samples is very light but 9 of the positive sera show strong recognition, not only in the 29–25 kDa region but also in the 55 kDa region

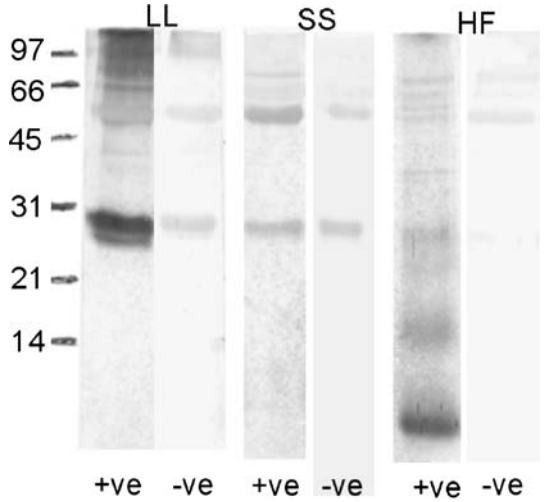

Fig. 7 Immunoblot of the laminated layer extract (*LL*), a sample of sheep serum (*SS*) and a sample of hydatid fluid (*HF*) probed for IgG_4 activity in a pool of positive sera (*+ve*) and a pool of negative sera (*–ve*). Note that there is strong recognition of the 29–25 kDa region (particularly at 25 kDa) in the laminated layer but much less recognition in this region in the sheep serum and hydatid fluid. Negative sera only show light staining in these regions

Discussion

The present results indicate that, as would be expected, the laminated layer taken from hydatid cysts in sheep contains a considerable amount of host proteins as identified by probing with anti-sheep IgG, which indicated that bands at 55 and 29 kDa were strongly recognised. Additional bands at 42, 38, 25 and 17 kDa were also recognised by anti-sheep IgG though it is not clear whether these bands represent breakdown products of immunoglobulin chains. Probing this layer with sera from infected humans, however, frequently indicates a strong antibody response, particularly of the IgG4 sub-class, to bands at the 55 kDa region and in particular the 29–25 kDa region. This is clearly not due to background activity of the probes used. It is, therefore, unclear whether this antibody response is against host or parasite antigens. If these bands do represent sheep heavy and light immunoglobulin chains, then we have a situation where some human patients with hydatid disease have circulating antibodies, which recognise sheep immunoglobulins. There are a number of possible explanations for this. Firstly, humans with hydatid disease could produce specific antibodies to sheep light and/or heavy immunoglobulin chains. Secondly, that bands in these regions could contain a mixture of parasite antigens and host proteins. Thirdly, that humans produce antibodies to parasite antigens, which share antigenic epitopes with sheep immunoglobulin chains.

The first of these explanations would be difficult to explain, i.e. why should humans infected with a parasite derived from dogs, produce antibodies against sheep immunoglobulins? This could be the case if there was some sort of auto-immunity in these people, which resulted in antibodies being produced against human immunoglobulins. There is no evidence, however, that auto-immunity is stimulated within hydatid patients (Colebrook and Lightowlers 1995).

Differences in the pattern of antibody binding between laminated layer extracts and sheep serum extracts, however, does not suggest that it is sheep immunoglobulins, which are being recognised because sera, which reacted strongly against the 29–25 kDa region of the laminated layer extract did not react strongly with a similar region in sheep serum. The possibility that there are parasite antigens in addition to host proteins in the 55 and 29–25 kDa region is a more likely situation and this is currently being investigated by fast protein liquid chromatography (FPLC) separation of parasite extracts. If such parasite components do exist, then they do not appear to be derived from the hydatid cyst fluid because the 25 kDa band was not recognised in this extract.

The possibility that the parasite is showing some sort of molecular mimicry by producing antigens, which share epitopes with sheep components, is interesting. A similar feature has been described with *T. crassiceps* where molecules resembling host immunoglobulins have been described (McManus and Lamsam 1990).

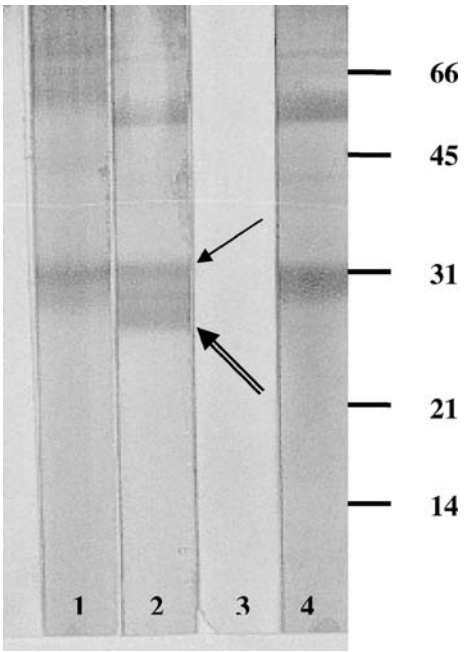

Fig. 8 Lectin blot of laminated layer extract indicating the presence of glycoproteins in the 29 (*leftwards arrow*)–25 (*leftwards black arrow*) kDa region with binding of SBA (*lane 1*), WGA (*lane 2*), DBA (*lane 3*) and ConA (*lane 4*). Results indicate the presence of *N*-acetyl-β-D-galactosamine, *N*-acetyl-β-D-glucosamine and α-methyl-D-manoside on the 29 and 27 kDa bands but only *N*-acetyl-β-D-glucosamine on the 25 kDa band

The results from this study indicate that, in at least some individuals with cystic hydatid disease, there is recognition of components within the laminated layer. The exact origin of these components is not clear but their level of recognition is unlikely to make them useful for diagnostics. Recognition of these components could, however, play a role in the subsequent development of the parasite and the nature of the immune response against it. The antigens recognised in the 25–29 kDa region of the laminated layer have a carbohydrate component and stimulate a dominant IgG4 response. Recognition of carbohydrate antigens in taeniid infections has received considerable attention lately (Miguez et al. 1996; Dai et al. 2001) and they may be important in influencing the regulation of the immune response (T cell activity) and its potential effect on parasite survival. In particular, they may influence a Th2-dominated response as indicated by a high level of IgG4 activity. There is significant evidence that a Th2-dominated response is more associated with progressive disease and a poor response to chemotherapy, whilst a Th1 response is more associated with a reduction in parasite growth and viability and successful chemotherapy (Ortona et al. 2003). In addition, laminated layer components from *E. granulosus* and *E. multilocularis* have been shown to have inhibitory effects on nitric oxide (a Th1 effector mechanism) production by macrophages (Steers et al. 2001; Andrade et al. 2004).

In conclusion, this study indicates, for the first time, that the isolated laminated layer of *E. granulosus* contains glycoproteins, which are antigenic and are recognised by some individuals with hydatid disease. The antigens appear to have no benefit in the diagnosis of the disease, but their recognition may be an important marker of disease status such as Th2-dominated response. Work is currently focused on matching anti-laminated layer responses with different cyst morphologies as defined by the WHO-OIE (2001) classification.

References

Andrade MA, Siles-Lucas M, Espinoza E, Arellano JLP, Gottstein B, Muro A (2004) *Echinococcus multilocularis* laminated layer components and the E14t 14-3-3 recombinant protein decrease NO production by activated rat macrophages in vitro. Nitric Oxide 10:150–155

Bigute J, Capron A, Tran-van-Ky P, D'haussy R (1962) Etude inmmunoelectrophoretique compare des antigens de divers helminthes. Comp Rend Acad Sci (Paris) 254:3600–3602

Colebrook LA, Lightowlers MW (1995) Lack of an association between hydatid disease and autoimmunity. Parasite Immunol 17:219–222

Coltorti EA, Varela-Diaz VM (1972) Penetration of host molecules into hydatidid cysts. Z Parasitenkd 48:47–51

Dai WJ, Hemphill A, Waldvogel A, Ingold K, Deplazes P, Mossmann H, Gottstein B (2001) Major carbohydrate antigen of *Echinococcus multilocularis* induces an immunoglobulin G response independent of alpha beta(+) CD4(+) T cells. Infect Immun 69:6074–6083

Dixon JB, Jenkins P (1995a) Immunology of mammalian metacestode infections I. Antigens, protective immunity and immunopathology. Helminthol Abstr 64:533–542

Dixon JB, Jenkins P (1995b) Immunology of mammalian metacestode infections II. Immunological recognition and effector function. Helminthol Abstr 64:599–613

Edwards GT (1982) Host IgG in equine hydatid cyst fluid. Ann Trop Med Parasitol 76:485–487

Gottstein B (1985) Purification and characterisation of a specific antigen from *Echinococcus multilocularis*. Parasite Immunol 7:201–212

Gottstein B, Echert J, Fey H (1983) Serological differentiation between *Echinococcus granulosus* and *E. multilocularis* in man. Z Parasitenkd 69:347–356

Gottstein B, Dai WJ, Walker M, Stettler M, Muller N, Hemphill A (2002) An intact laminated layer is important for the establishment of secondary *Echinococcus multilocularis* infection. Parasitol Res 88(9):822–828

Hulsmeier AJ, Gehrig PM, Geyer R, Sack R, Gottstein B, Deplazes P, Kohler P (2002) A major *Echinococcus multilocularis* antigen is a mucin-type glycoprotein. J Biol Chem 277:5742–5748

McManus DP, Lamsam S (1990) *Taenia crassiceps* surface immunoglobulins: parasite- or host-derived? Parasitology 101:127–137

Miguez M, Baz A, Nieto A (1996) Carbohydrates on the surface of *Echinococcus granulosus* protoscoleces are immunodominant in mice. Parasite Immunol 18:559–569

Ortona E, Rigano R, Buttari B, Delunardo F, Ioppolo S, Margutti P, Profumo E, Teggi A, Vaccari S, Siracusano A (2003) An update on immunodiagnosis of cystic echinococcosis. Acta Trop 85:165–171

Rickard MD, Williams JF (1982) Hydatidosis/cysticercosis: immune mechanisms and immunization against infection. Adv Parasitol 21:229–296

Rogan MT, Craig PS (1997) Immunology of *Echinococcus granulosus* infections. Acta Trop 67:7–17

Russi S, Siracusano A, Vicari G (1974) Isolation and characterisation of a blood P1 active carbohydrate antigen of *Echinococcus granulosus* cyst membrane. J Immunol 112:1061–1069

Steers NJ, Rogan MT, Heath S (2001) In Vitro susceptability of hydatid cysts of *Echinococcus granulosus* to nitric oxide and effect of the laminated layer on nitric oxide production by peritoneal macrophages. Parasite Immunol 23:411–417

Taherkhani H (1998) Studies on the antigenicity of the laminated layer of *Echinococcus granulosus*. Ph.D. thesis, University of Salford, UK

Varela-Diaz VM, Torres JM (1977) Antigenic characterisation of *Echinococcus granulosus* cysts. Boll Ist Sieroter Milan 56:303–309

WHO-OIE (2001) In: Eckert et al (ed) Manual on echinococcosis in humans and animals: a public health problem of global concern. WHO, Geneva